

WINDOW consortium

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WINDOW consortium: A Path Towards Increased Therapy Efficacy Against Glioblastoma

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ABSTRACT

Glioblastoma is the most common and malignant form of brain cancer, for which the standard treatment is maximal surgical resection, radiotherapy and chemotherapy. Despite these interventions, mean overall survival remains less than 15 months, during which extensive tumor infiltration throughout the brain occurs. The resulting metastasized cells in the brain are characterized by chemotherapy resistance and extensive intratumoral heterogeneity. An orthogonal approach attacking both intracellular resistance mechanisms as well as intercellular heterogeneity is necessary to halt tumor progression. For this reason, we established the WINDOW Consortium (Window for Improvement for Newly Diagnosed patients by Overcoming disease Worsening), in which we are establishing a strategy for rational selection and development of effective therapies against glioblastoma. Here, we overview the many challenges posed in treating glioblastoma, including selection of drug combinations that prevent therapy resistance, the need for drugs that have improved blood brain barrier penetration and strategies to counter heterogeneous cell populations within patients. Together, this forms the backbone of our strategy to attack glioblastoma.

BACKGROUND AND CHALLENGES

Classification of glioblastoma and inter-patient heterogeneity

Gliomas are the most common malignancies of the central nervous system (CNS). Most glioblastomas (GBMs) arise *de novo* without any sign of a less malignant precursor (Ohgaki and Kleihues, 2013). Primary GBMs typically occur at an average age of 62 years, rapidly progress and have an extremely poor prognosis. However, 10% of GBMs progress from low-grade diffuse astrocytomas or anaplastic astrocytomas (**Figure 1A**). These “secondary” GBMs occur at an average age of 45 years and offer a slightly better prognosis than primary lesions. According to the WHO 2016 classification, gliomas are defined based on mutations in the IDH1 gene with/without chromosomal 19q loss (summarized in **Figure 1B**, taken from Verhaak 2016). Astrocytic gliomas are classified based on histologic criteria from lower grade lesions (grades II–III) to high-grade (grade IV) malignancies (WHO 2016; previous classifications given in Louis et al, 2010; Zhu and

Parada, 2002; Weller et al, 2005). Originally, the Cancer Genome Atlas Network (TCGA, Verhaak et al, 2010) defined molecular signatures within GBM (grade IV), which were distinguishable based on expression of lineage markers. These subgroups are termed “classical”, “mesenchymal”, “proneural” and “neural”, although existence of the latter group is debated (Sidway 2017). The proneural subtype correlates with better prognosis whereas the mesenchymal and classical subtypes correspond to poor prognosis (Lin et al, 2014). These quantitative relationships, based on WHO criteria, molecular features and histology are summarized in **Figure 1**.

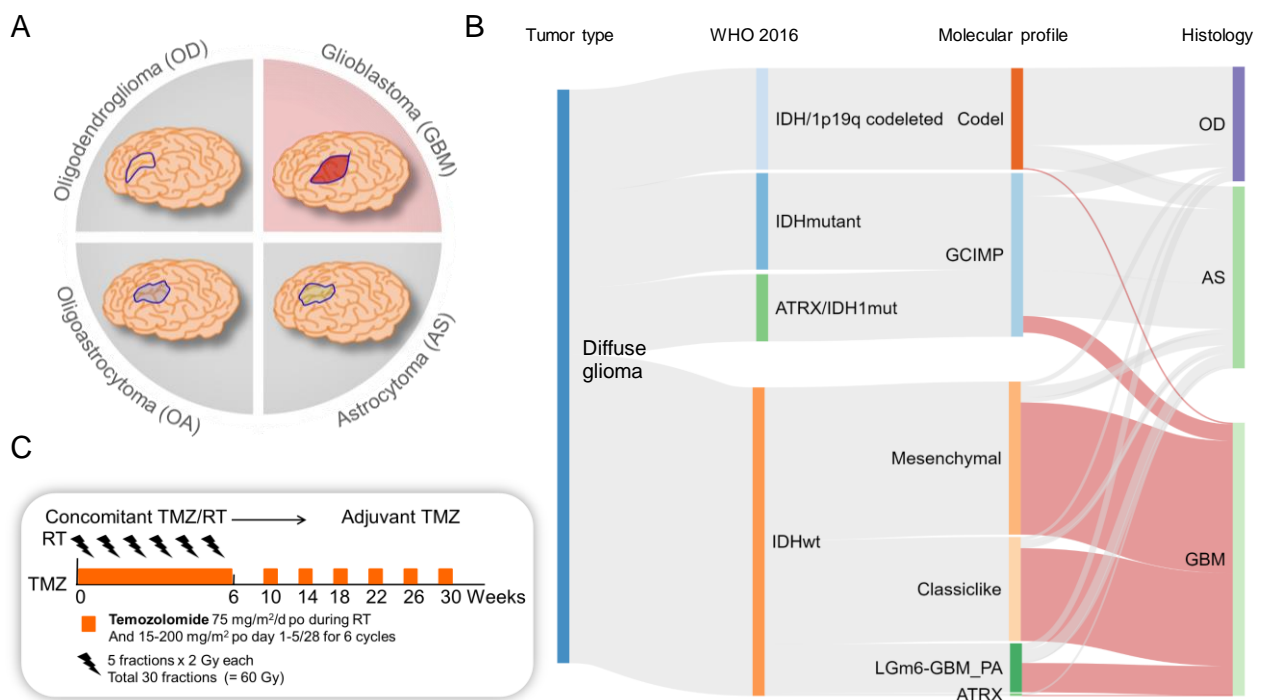


Figure 1. (A) Circos plot showing the histological classification system as used in the clinic until recently. (B) Sankey plot showing the quantitative relation of different adult gliomas (based on 1,122 patients in the TCGA database www.cbioportal.org, see also Carelli et al 2016 and Weller et al 2017), grouped by the WHO 2016 classification (IDH mutated; IDH/ATRX comutated; 1p19q codeletion); molecular profile (Mesenchymal, Classic like, LGM6-GBM_PA [LGGs in the third methylation cluster of IDH-wild type tumours as pilocytic astrocytoma-like], GCIMP; and histological class: astrocytoma (AS), oligodendroglioma (OD) and glioblastoma (GBM). (C) Current treatment of GBM after maximal safe surgical resection. After surgery, RT is continued for 6 weeks combined with TMZ treatment. Subsequently, adjuvant TMZ five days per week is indicated for at least six months. po, per os (oral administration).

Current clinical trials are not focused on GBM driver mutations

The standard treatment of GBM is based on the international EORTC-study (Stupp et al. 2005), consisting of maximal safe surgical resection, followed by six weeks of radiotherapy (RT) and concomitant Temozolomide (TMZ) chemotherapy with subsequent adjuvant TMZ chemotherapy for six months, **Figure 1C**. Compared to RT alone, with a median survival time of 12.1 months, the combination of RT plus TMZ increases median overall survival time (OS) by 2.5 months to 14.6 months (Weller et al, 2016; Stupp et al, 2009; 2005). A group of patients displaying silencing of the MGMT-gene promoter via DNA methylation, have repressed DNA damage repair and is therefore more sensitive to the alkylating effect of TMZ (Hegi et al, 2005). In clinical practice, TMZ is administered regardless of the patient's promoter methylation status.

Many efforts are underway to improve the outcome of the current standard therapy, attacking driver as well as non-driver targeted therapies (graphically summarized in **Figure 2A**). Currently, there are more than 225 ongoing trials in GBM, of which over 60% include systemic therapy, comprising chemotherapy/DNA damaging compounds (23%), immunotherapy (13%), VEGF targeted therapy (8%) and PI3K targeted therapy (3%), shown in Sankey plot in **Figure 2B**.

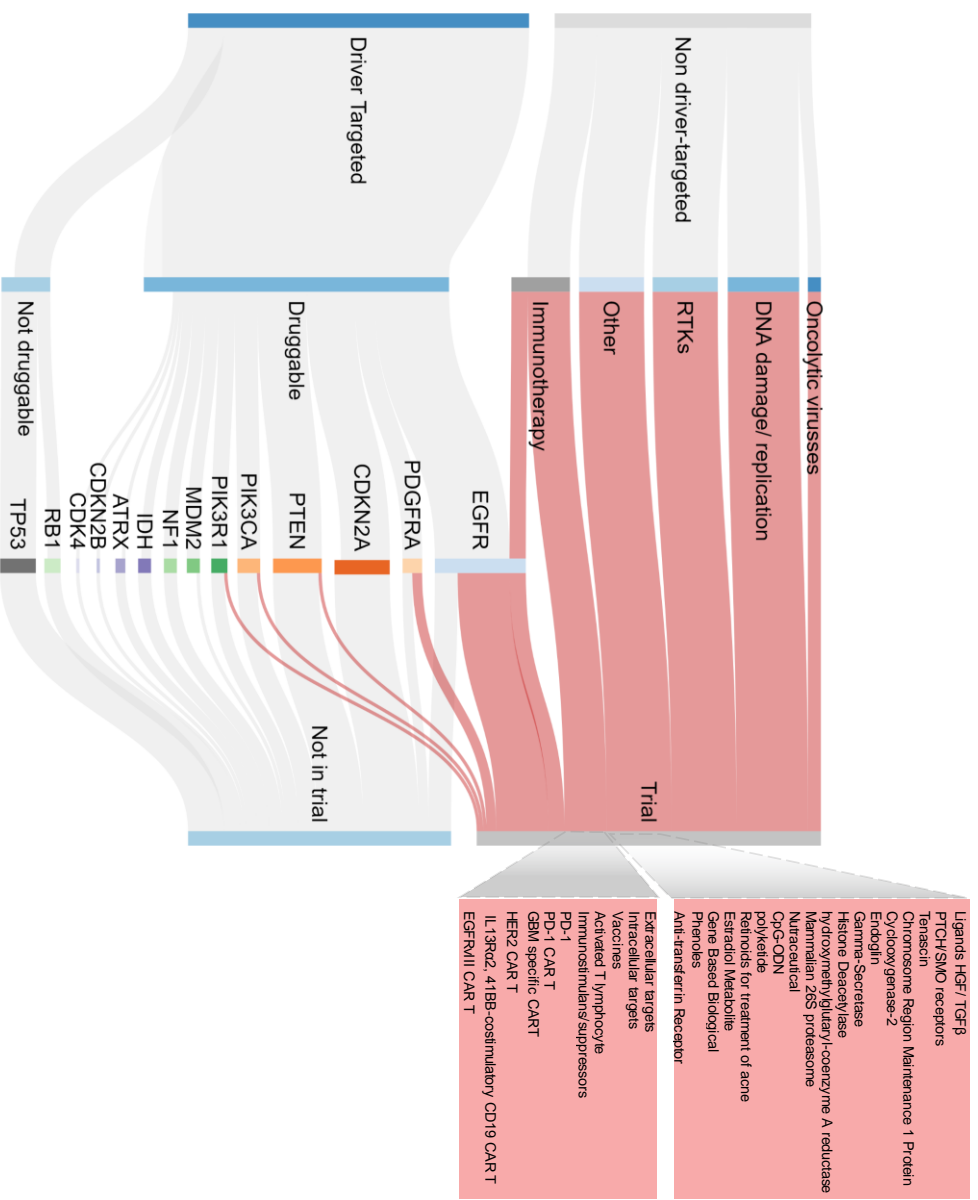
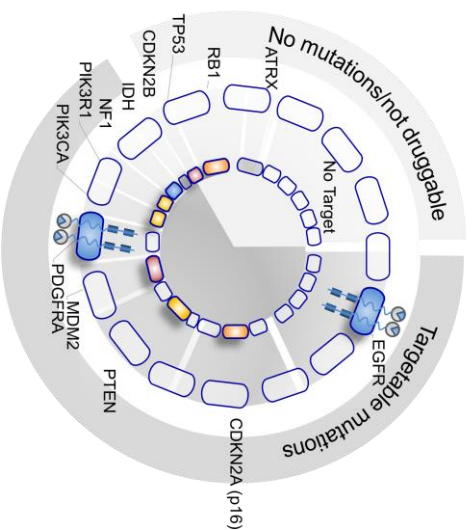
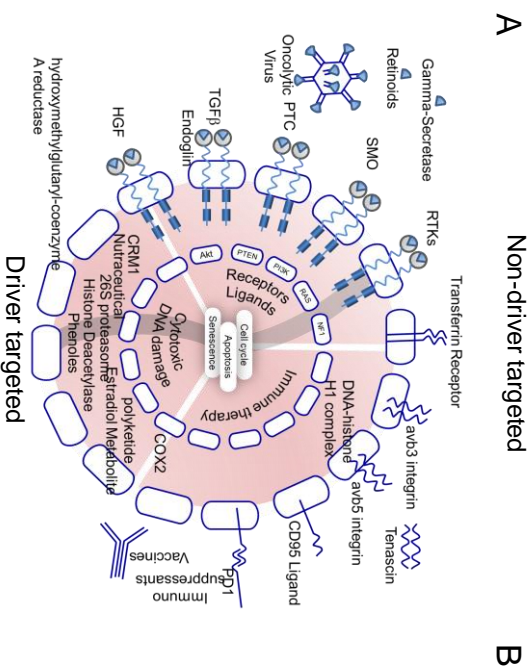


Figure 2. Driver versus non-driver focussed therapies against GBM in clinical trials (ClinicalTrials.gov). (A) Circos plots showing non-driver targets and driver targets of currently applied clinical trials against GBM. Cellular location of targets is shown as extracellular, membrane bound or intracellular. Non-driver genes are classified into immune targets, receptors and receptor ligands; and cytotoxic/DNA damaging targets. The driver mutation circos plot is divided in targetable mutations, absence of druggable mutations and non-druggable driver mutations. (B) Sankey plot showing the quantitative relation of the number of trials towards each target in ongoing clinical trials. Non-driver targets (upper portion) as well as driver targets using FDA approved mutation specific small molecules (lower portion) are shown. Note that almost no approaches to driver targets are currently in clinical trials. Abbreviations: RTK, receptor tyrosine kinase; SMO, smoothened Shh receptor; Ptc, Patched Shh receptor; TGF β Transforming growth factor beta; HGF, Hepatocyte growth factor; CRM1, chromosome region maintenance 1; COX2, Cyclooxygenase 2; PD1, Programmed cell death protein 1; EGFR, Epidermal growth factor receptor, ATRX, Alpha Thalassemia/Mental Retardation Syndrome X-Linked gene; RB1, retinoblastoma 1 protein; TP53, tumour protein p53; p15/CDK2NB, cyclin-dependent kinase inhibitor 2B; IDH1 Isocitrate dehydrogenase homolog 1; NF1, Neurofibromatosis type 1; PIK3R1 Phosphatidylinositol 3-kinase regulatory subunit alpha; PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; PDGFRA, Platelet-derived growth factor receptor A; MDM2, Murine double minute 2; PTEN, Phosphatase and tensin homolog; p16/CDK2NA, cyclin-dependent kinase inhibitor 2A; CDK4, cyclin dependent kinase 4.

Although 70% of GBMs contain tumor driver mutations that are targetable by therapy (**Figure 2**, Carelli et al, 2016), in current clinical trials there is no focus on these driver mutations, except for EGFR- targeted therapy (11% of clinical trials, **Figure 2B**). Trials aimed at driver-mutations have been largely unsuccessful (for instance NCT00187486, NCT0062243 and NCT00671970 aimed at EGFR inhibition), possibly because of poor penetrance of drugs into the CNS through the blood brain barrier [BBB; (Oberoi et al, 2013; Tang et al, 2012; Porta et al, 2011)].

Here, we address the challenges associated with the efficient treatment of GBM through combination therapy. We see these as: (1) identification of drugs that have desired BBB penetration, (2) addressing whether combination-therapies are of benefit in targeting intratumoral heterogeneity and (3)

overcoming drug resistance. Based on this landscape, we outline the strategy adopted by the WINDOW consortium for the selection and further development of effective therapies against GBM.

Challenge 1: The BBB and perivascular/perineural microinvasion – The BBB is the natural barrier that prevents toxins from reaching the brain. It is a major obstacle to brain tumor therapy, preventing the delivery of most chemotherapeutic and targeted agents to the tumor location (e.g. Sminia and Westerman 2016; van Tellingen et al, 2015). Since most drugs have been developed for applications outside the brain and therefore selected for low BBB penetration to avoid neurotoxicity, most FDA approved drugs have poor target engagement in the brain.

Gliomas show perivascular and perineural microinvasion (Cuddapah et al, 2014; Gritsenko et al, 2012; Montana et al, 2011; Farin, et al, 2006). Glioma tumor cells can form multicellular networks connected through branched filamentous protrusions connecting cells, or epithelial-like linear adherent junctions between directly adjacent cells (Friedl et al, personal communication). Therefore, individual tumor cells are localized distantly from the bulk of the tumor (Sherriff et al, 2013) and might be more difficult to reach both therapeutically as well as surgically.

For many years, different strategies have been investigated to facilitate BBB penetration by chemotherapeutics, reviewed in Upadhyay, 2014 and Lu et al, 2014. These include the use of 1) non-invasive techniques, such as radiotherapy (Trnovec et al, 2016), ultrasound and microbubbles (Lamanauskas et al, 2013; Escoffre et al, 2013; Rachlin et al, 2013; Chu et al, 2016), and biological approaches via cell penetrating peptides and viral vectors (Sidaway, 2018); 2) invasive techniques, such as convection enhanced delivery (reviewed by Vogelbaum and Aghi, 2015), and 3) alternative routes such as intranasal application, bypassing the cardiovascular system (reviewed by Peterson et al, 2014).

The high prevalence of brain metastasis from the lung, breast and melanoma, makes it attractive to develop compounds that target driver mutations specific for these tumor types, leading to the development of potent kinase inhibitors that do cross the BBB. These include compounds targeting driver mutations such as Osimertinib (targeting EGFR T790M) and Lorlatinib (targeting ALK mutations), as well as the radio-sensitizing drug AZD0156 (ATM) and the PI3K/AKT/mTOR pathway inhibitor GDC-0084. GBM is rarely the main therapeutic focus, due to its relatively small market size. However, because drug discovery

is a long-term endeavour and an extremely expensive one (estimated that a single drug can take over 10 years and cost more than £500M to develop) (Austin, 2017), drug repurposing (i.e. the identification of new therapeutic uses for existing drugs), is the only route currently available as a solution to this challenge. To date, there has been no systematic evaluation of the already available approved drugs against GBM.

Challenge 2: Drug resistance and intratumoral molecular heterogeneity

GBM is characterized by intratumoral heterogeneity in which subpopulations of cells have distinct features, largely consisting of (1) gene copy number variations as shown for PTEN, TP53 and MDM4 (Sottoriva et al, 2013; Johnson et al, 2014; Meyer et al 2015), (2) clonal ploidy differences (Stieber et al, 2014; Johnson et al. 2014; Meyer et al, 2015), (3) extrachromosomal DNA elements (deCarvalho et al, 2018; Turner et al, 2017), (4) signalling heterogeneity as a result of mosaic receptor tyrosine kinase activity (Snuderl et al, 2011; Little et al. 2012; Szerlip et al. 2012) including structural variants of EGF receptor (Francis et al, 2014; Meyer et al, 2015), and (5) lineage heterogeneity, i.e. GBM cells express markers of lineage derivatives of stem/progenitor cells such as proneural, neural as well as astroglial genes (Verhaak et al, 2010; Phillips et al, 2006; Al-Mayhany et al, 2011; Sottoriva et al. 2013; Patel et al, 2014; Kenney-Herbert et al, 2015; Piccirillo et al, 2015a; Piccirillo et al, 2015b; Wang et al, 2016, Meyer et al, 2015). The different forms of cellular heterogeneity are summarized in **Figure 3A**.

The sensitivity of individual tumors towards drugs can vary due to intrinsic resistance which is pre-existing or acquired as a result of drug therapy (Sequist et al, 2011; Garrett et al, 2011; Prahallad et al, 2012; Wilson et al, 2012). Drug resistance, intrinsic or acquired, is affected by intratumoral heterogeneity due to genetic or phenotypic heterogeneity (the mechanisms are listed in **Figure 3B**). Preclinically, intratumoral subpopulations display a differential response to therapeutics (Meyer et al, 2015; Hägerstrand et al, 2011; Heo et al, 2014; Saito et al, 2014; Lee et al, 2017; Lan et al, 2017). Moreover, a re-transplanted tumor seems to retain its intrinsic resistance, indicating that there is a population with a long term tumor initiation or drug resistance potential (Lan et al, 2017). These studies implicate intrinsic cell populations in each GBM patient exhibiting pre-existing resistance to therapy (Meyer et al, 2015). In the clinic, radiation resistance is observed frequently in relapsed GBMs (Hochberg and Pruitt, 1980; Kelley et al, 2016; Fidoamore et al, 2016). Several alternative mechanisms have been found to underlie therapy resistance.

For instance, EGFR inhibitor resistance may occur as a consequence of loss of extrachromosomal mutant EGFR DNA, promyelocytic leukemia (PML) gene expression, PTEN phosphorylation status, PDGFR upregulation, ERBB4 activation, AMPK levels or IL-6 upregulation (Nathanson et al, 2013; O'Rourke et al, 2017; Iwanami et al, 2013; Fenton et al, 2012; Akhavan et al, 2013; Donoghue et al, 2018; Guo et al, 2009; Zanca et al, 2017).

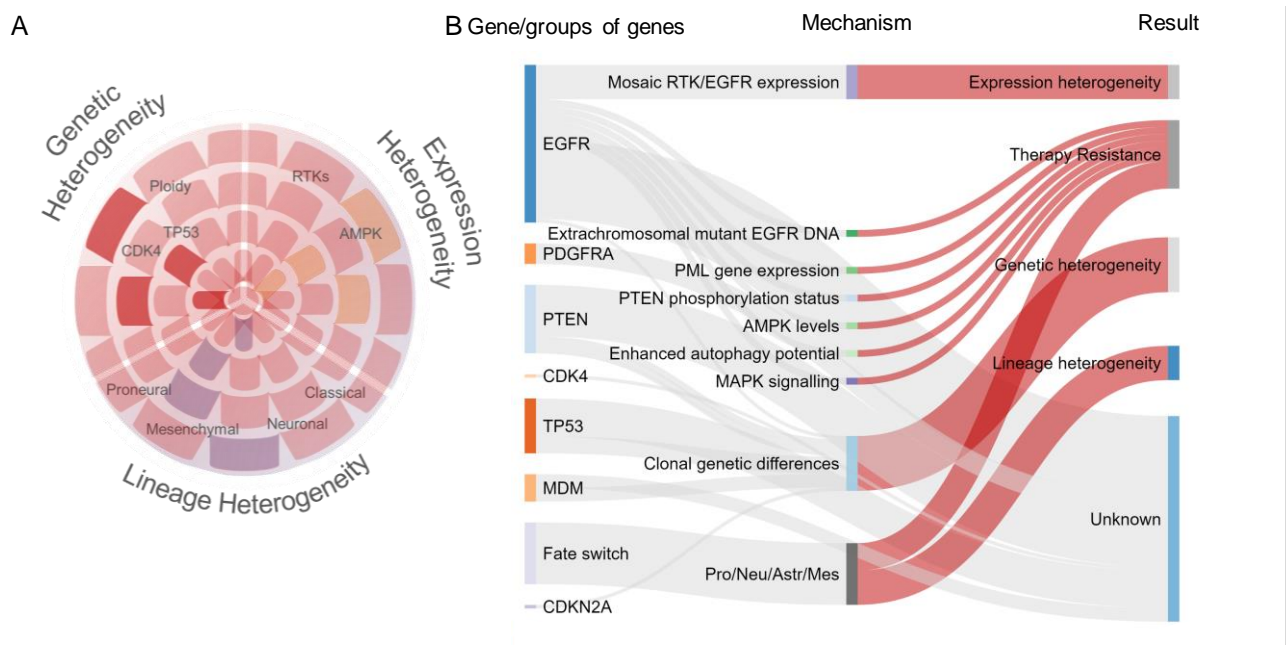


Figure 3. Resistance and heterogeneity mechanisms of GBM. (A) Circos plot showing different molecular mechanisms underlying the generation of cellular heterogeneity; (B) Sankey plot quantitatively summarising the causes of heterogeneity and drug resistance involving driver targets in GBM. Targets that are frequently mutated are shown in color. PML, promyelocytic leukemia; AMPK, 5' adenosine monophosphate-activated protein kinase; MAPK, Mitogen Activated Protein Kinase.

Challenge 3 Combination therapies against heterogeneous populations

Since intratumoral heterogeneity and drug resistance can occur simultaneously, a promising approach might be to combine targeted drugs that hit several survival mechanisms at once for different cell populations. This could provide a synergistic (i.e. more than additive) effect and prevent therapy-resistance. However, identification of these synergistic combinations has thus far only been possible in an empirical setting (by experimentally testing all combinations). A major challenge is therefore to match GBM vulnerabilities to effective drug combinations.

Tumors are dependent on a limited number of molecular mechanisms for their survival and proliferation (Hanahan and Weinberg, 2011; Wang et al, 2015). Combination therapy enables simultaneous targeting of these crucial mechanisms. In addition, for personalized cancer treatment, a focus on drug/irradiation or drug/drug combinations is particularly appealing since enhanced efficacy can be assessed for each radiation-dose or drug-concentration window.

Strategies for selecting synergistic and effective drug combinations (Dancey and Chen, 2006; Day and Siu, 2016) can be based on several precepts, including: (1) maximal target inhibition, achieved by synergistic drug combinations that hit the same target, especially important in the case of oncogene addiction. Drug resistance might also be avoided by this approach as well as other novel approaches (Barzev et al, 2017; Li et al, 2016; Zhitomirsky et al, 2016); (2) maximal pathway inhibition. In a similar manner, maximal pathway inhibition can be used to suppress an entire pathway by inhibiting it at multiple levels. An example of this strategy is the FDA approved combination of the BRAF inhibitor vemurafenib and the MEK inhibitor cobimetinib in BRAFV600 mutated melanoma which results in maximal pathway inhibition in cases where the tumor is dependent on the BRAFV600 mutation (Larkin et al, 2014). Also, within GBM radiotherapy and PARP-inhibition have shown synergy (Lesueur et al, 2018); (3) feedback inhibition between and within pathways. In many cases this affects upstream pathway activation through downstream targets of the initial inhibited pathway (Sun and Hobor et al, 2014; Sun and Wang et al, 2014; Rozengurt et al, 2014); and finally (4) synthetic lethality (feed forward) inhibition, in many cases affecting inhibition of two parallel pathways downstream of activated oncogenes (Croesmann et al, 2018). For feedback inhibition and synthetic lethality approaches, information on underlying therapy-resistance is scarce, and direct targets are difficult to identify, rendering the rational design of pathway combinations difficult.

AIMS OF THE WINDOW CONSORTIUM

General aims of the consortium

Within the WINDOW consortium, our aim is to provide a solution to these challenges by examining the links between intratumor molecular heterogeneity, prediction of *in vitro* therapeutic drug combination and testing, as well as emerging clinical data, areas which must be addressed for successful development of

effective therapeutics. Our overall objective is to create a validated patient-centred platform based upon integration of patient-derived cell systems, detailed genetic analysis of tumor cell population, together with their treatment with the most effective combination therapies. At a more immediate level, we will develop a collaborative pipeline to enable patient-stratified treatments with the most effective combinations of FDA approved drugs, thereby offering a Window for Improvement for Newly Diagnosed patients thereby Overcoming Disease Worsening (WINDOW).

WINDOW specific aim 1: Repurposing of clinically approved drugs

To be able to repurpose FDA approved drugs against GBM, drugs must combine traditional qualities, such as optimal systemic absorption, distribution, metabolism, and excretion (ADME), with enhanced CNS bioavailability. Selection criteria are therefore: (1) molecular mode of action [i.e. target specificity], (2) experimental proof of efficacy, (3) ADME characteristics, (4) documented CNS penetrance and (5) toxicology including the absence of neural side-effects.

Most FDA approved drugs will never reach the brain because they were selected based on inability to cross the blood brain barrier. In addition, most drugs are substrates for efflux pumps and are actively pumped out of the brain through ABC transporters such as P-gp and ABCG2. Recent development of drugs against metastasized from the lung, skin and breast tumors have provided important information how to design drugs that have optimal characteristics to reach the brain tumor. Primary brain tumor have suffered from a lack of clinically relevant drugs targeting brain specific lesions. Nevertheless, we show here how to use data from non-primary brain tumor fields to select drugs that might be lead drugs for application against brain tumors. A first step in this process has been to curate GBM drug information in a form that is readily accessible to all preclinical GBM researchers. This has now been achieved through the establishment of the GBM Drug Bank (www.gbmdrugbank.com; Svensson et al 2018). This resource includes all FDA approved drugs suitable for repurposing that are active in GBM preclinical models.

WINDOW specific aim 2: Overcoming drug resistance through combination therapies

In the WINDOW project, we have developed a novel strategy to identify effective drug-combinations using a topology-based approach which we call the drug-atlas (Narayan et al, submitted). This atlas is built from

drug-response encyclopedias and can be considered as a framework of therapeutic action and therefore can be interpreted as a drug-vulnerability landscape of cancer. The methodology is based on the finding that most tumors contain multiple independent survival/proliferation mechanisms. This novel rational and generalizable strategy opens the door to unforeseen personalized multi-drug combination approaches.

Some of the drug combinations identified with the drug atlas will show a lack of response in a number of cell lines. For this we will apply CRISPR/CAS9 technologies to elucidate the molecular basis for the lack of therapeutic response (Tzelepis et al, 2016). We will (1) identify genetic vulnerabilities of these clonally derived cell lines and to determine the mechanisms that are responsible for the lack of response therapy. Clonally derived glioblastoma cell lines that show drug resistance are infected with a CRISPR knock out library. Subsequently, next gen sequencing of the CRISPR cassettes will reveal which CRISPR constructs are lost upon exposure to the combination therapy and hence identify the genes responsible for the sensitization to the drug combination. This drop out screen methodology will reveal the sensitivities of the drug resistant cell lines, which will differ from the control cell line. Finally, drugs can be chosen that hit the identified protein or process as directly as possible. In addition, drug resistance mechanisms to monotherapy or combination therapy can be identified using CRISPR screening technologies.

Two major limitations of combination therapy with targeted drugs are the narrow time window wherein drugs can or need to be delivered, and the narrow therapeutic window between enhanced tumor kill and toxicity. Examples of drug combination-induced toxicities are shown in **Table 1**.

Table 1
Examples of accumulating toxicities

Challenge	Drug combination	Tumour type	Clinical phase	Outcome	Reference
Target engagement & PK/PD	Sirolimus + erlotinib	GBM	Phase II	No improvement over control	Reardon et al, 2010
	Cetuximab or panitumumab to bevacizumab and chemotherapy	Colon	Phase III	No improvement over control	Hecht et al, 2009; Tol et al, 2009
Toxicity	Bevacizumab, irinotecan, temozolomide	GBM	Phase II	Hematological toxicity	Peters et al, 2015
	Bevacizumab (VEGF inhibitor), temozolomide	GBM	Phase II	Blood pressure, Hematological toxicity	Reyes Botero et al, 2018
	Onartuzumab (c-MET)	GBM	Phase II	Peripheral edema, Asthenia	

	bevacizumab (VEGF inhibitor)					
	Bevacizumab (VEGF inhibitor) + sunitinib	Renal cell carcinoma	Phase I	Vascular/hematological toxicities		Feldman et al, 2009*
	Temsirolimus (mTOR inhibitor) + sunitinib	Renal cell carcinoma	Phase I	Skin/hematological toxicities		Patel et al, 2009*
	Everolimus (MTORC1) combined with exemestane	Breast cancer	Phase III	Stomatitis, intestinal, hematological toxicities		Baselga et al, 2012
	MEK inhibitor combined with AKT inhibitor	Various	Phase II	Intestinal, skin		Tolcher et al, 2015
	Ipilimumab combined with nivolumab	Melanoma	Phase III	Intestinal, skin		Larkin et al, 2015
	BRAF inhibition combined with MEK inhibition	Melanoma	Phase II	Fever		Flaherty et al, 2012

*Taken from Day and Siu Genome Medicine (2016) 8:115

Additive toxicities can be avoided by using drug combinations that have different toxicity patterns. For example, in the combination with irradiation, the crucial issue is the scheduling and administration of targeted and radiosensitizing agents. To predict possible toxicities, we will generate a Toxicity Atlas, to enable selection of drug-combinations with complementary toxicities and prevent accumulated side effects.

Future Perspectives

By using our WINDOW consortium approach, we aim to overcome issues that currently prevent an effective therapeutic strategy against GBM. These issues concern intratumoral heterogeneity and identification of more effective therapies targeting the heterogeneous tumor populations characteristic of GBM, initially focussing on the repurposing of FDA-approved small molecule drugs. Recognizing the fact that many of the current set of FDA-approved drugs were developed with non-CNS penetrance as an objective, the WINDOW program has the longer-term aim of re-developing approved drugs that show efficacy in preclinical models, through industry-proven lead optimization approaches.

Based on the high prevalence of brain metastases from lung cancer, breast cancer and melanoma, industry research is leading to the development of kinase inhibitors for treating brain metastases of other solid tumors, including driver-targeted compounds such as Osimertinib, AZD3759, NT113, Lorlatinib, GDC-

0084, as well as the radio-sensitizing drug AZD0156. An additional focus could be on inhibitors showing polypharmacology (Knight et al., 2010), in which either a single drug or a combination of drugs can restrict or even gridlock adaption mechanisms of the tumor.

Many of these new approaches could be tested in preclinical models recapitulating the inter- as well as intra-patient heterogeneity to enable further dissection of the molecular basis of resistance development in the context of the host-tumor interaction, as well as direct tumor toxicity. A better mechanistic understanding of the toxicity of drug combinations and/or drug polypharmacology will assist in prioritizing candidate therapies. Moreover, activation of the immune system might further enhance the effects of our strategy. The objective of the WINDOW Consortium is to combine these paradigms, to deliver new and effective therapeutic strategies for GBM.

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